MicroRNAs as biomarkers for ischemic heart disease

Van Aelst; miR and CAD

Lucas N.L. Van Aelst, MD[1, 2]; Stephane Heymans, MD PhD[1, 3, 4]

[1]Center for Molecular and Vascular Biology, University of Leuven, Herestraat 49, Box 911, 3000 Leuven, Belgium.
[2]Department of Cardiology, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium.
[3]Center for Heart Failure Research, Cardiovascular Research Institute Maastricht (CARIM), University Hospital Maastricht, 6229 HX Maastricht, the Netherlands.
[4]Interuniversity Cardiology Institute Netherlands (ICIN), Royal Netherlands Academy of Sciences, Utrecht, the Netherlands.

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Corresponding author:
Stephane Heymans, MD PhD
Cardiovascular Research Institute Maastricht, Maastricht University
Department of Cardiology, University Hospital Maastricht
Universiteitssingel 50, room 3214
P. Debyelaan 25
6202 AZ Maastricht
The Netherlands
Fax: +31 (0)433871055
Phone: +31 (0)433882949 , +31 (0)433882950
E-mail: s.heymans@maastrichtuniversity.nl

Abstract

MicroRNAs (miRs) are short, non-coding RNAs that function as posttranscriptional inhibitors of mRNA translation to protein. They are essential for normal development and homeostasis. Dysregulated expression patterns both cause and result from disease states. Generally studied as intracellular mediators, miRs can be isolated from body fluids and exhibit remarkable stability to degradation. These features, in combination with their tissue specificity, make miRs attractive candidates as blood-derived biomarkers for coronary artery disease (CAD), the most frequent cause of death worldwide.

The use of miRs as biomarkers in both symptomatic and asymptomatic CAD and the influence of conventional cardiovascular risk factors and CAD treatment on their circulating levels is the topic of this review. To conclude, it highlights the remaining hurdles to tackle before this promising application of miRs can enter into routine clinical practice.

Key words: microRNA – coronary artery disease – biological markers – myocardial infarction – heart failure.

Introduction

MicroRNAs (miRs) are single-stranded, phylogenetically conserved, non-coding RNA molecules of ~22 nucleotides which inhibit mRNA translation to protein by interacting with its 3' untranslated region[1]. They are crucially involved in many aspects of cardiac development, homeostasis and pathobiology[2]. Although miRs function as endogenous intracellular regulators of mRNA translation, they can be detected in circulating blood or its components in a remarkably stable form and therefore should be considered disease biomarkers, for instance in cardiovascular diseases[3].

Ischemic heart disease virtually always results from coronary artery disease (CAD). It is the most frequent cause of death worldwide and accounts for seven million deaths annually. Heart failure, silent ischemia, angina pectoris (AP) and myocardial infarction (MI) are its main clinical manifestations[4]. Acute coronary syndrome (ACS) is a collective noun for AP and MI, manifesting clinically as crushing chest pain with accompanying repolarization changes on the electrocardiogram (ECG). In the acute care setting, differentiating ACS and non-cardiac causes of chest pain remains a diagnostic challenge[5]. However, a correct early diagnosis is associated with improved outcome[6], underscoring the need for a reliable biomarker for the early detection of cardiomyocyte death. Current guidelines advocate the use of the MB fraction of creatine kinase (CK-MB) or cardiac troponins T and I (TnT and I) released in the circulation by damaged cardiomyocytes to diagnose MI[7] and stress the importance of serial measurements to "rule in" or "rule out" MI, as Tn or CK-MB elevations might be delayed[8]. This low sensitivity during the first 6 hours (h) after the onset of chest pain is a major drawback of contemporary assays, partially resolved by the introduction of high sensitive (hs) Tn assays, with a sensitivity of 3 hours[8].

In ischemic heart failure (HF), CAD hampers the heart to meet the metabolic demands of the body[9]. Typical symptoms suffice to make a diagnosis of HF, but identification of CAD as

its etiology requires additional invasive tests[10]. A reliable biomarker would obviate the need for these potentially harmful investigations.

This report summarizes the current knowledge on the use of circulating miRs as diagnostic and prognostic biomarkers in asymptomatic and symptomatic CAD. It also describes the effect of CAD risk factors and different medications typically initiated after ACS on circulating miR levels. To conclude, we discuss their value in relation to existing biomarkers and indicate remaining gaps to bridge before their implementation in routine clinical practice (Table 1,2; Figure 1).

MiR stability in blood and blood-derived products

The existence of circulating miRs notwithstanding high plasma RNase activity suggests their shielding from enzymatic degradation in addition to their resistance to physical conditions inducing immediate degradation of free RNA (e.g. repeated freeze-thawing, prolonged exposure to room temperature)[3]. Accumulating evidence shows that lipid vesicles, lipoprotein complexes and proteins protect circulating miRs.

In contrast with initial beliefs, lipid vesicles carry only a minority of circulating miRs[11]. Apoptotic bodies are the largest of these lipid vesicles (up to 4 μm), shed when cells undergo apoptosis. Microvesicles (100 nm – 1 μm) bud from the plasma membrane under physiological and pathological conditions as packages containing a set of biological signals modulating cellular behaviour at a distance[12]. Exosomes are small vesicles (50 – 90 nm), derived from the endosomal compartment and released when multivesicular bodies fuse with the plasma membrane[12,13]. Higher levels of distinct miRs in exosomes than in their donor cells suggest an active mechanism of miR loading into multivesicular bodies and exosomes[14,15]. Recently, also high-density lipoproteins (HDL) were implicated in shielding circulating miRs[16]; hereby, neutral sphingomyelinase regulated miR export to

HDL and scavenger receptor class B type I mediated miR delivery to recipient cells. However, the majority of circulating miRs are bound to RNA-binding proteins. The exact release mechanism is unknown, but may be mere passive release upon disruption of the plasma membrane. Argonaute 2 (Ago2), which acts intracellularly in concert with miRs to form an RNA-induced silencing complex, as well as nucleophosmin 1 (NPM1) have been implicated as protein carriers for circulating miRs[11,17,18].

The early release of cardiomyocyte-enriched miR-1, miR-133a/b and miR-208 in ACS suggests 'leakage' from necrotic cardiomyocytes[19,20], with the slow rise of miR-499 attributable to slower release of its carrier protein. However, in this regard, the repeated inability to detect other cardiomyocyte enriched miRs such as miR-24 and miR-30c in blood or its components upon cardiac ischemia is remarkable and suggests active degradation and release mechanisms such as apoptosis and resulting release of apoptotic bodies. Furthermore, depletion of ATP with resultant increase in intracellular calcium concentration leads to augmented extrusion of exosomes by cardiomyocytes in vitro[21]. While activated or apoptotic endothelial cells release microvesicles respectively apoptotic bodies in the circulation[22-24], primary human fibroblasts primarily release non-vesicle associated miRs following serum deprivation in vitro. This fragmentary combination of in vitro and in vivo observations on the biochemical composition and the timing of release of extracellular miRs only gives a rough and incomplete sketch of the exact proportion of vesicle-associated and non-vesicle-associated circulating miRs (and their respective subfractions) in various disease states and healthy individuals. Additional research is essential to address these questions and obtain a detailed and comprehensive picture, which could permit the isolation of miRs specifically enriched in selected subfractions, certain cell types or at specific time points, thus creating more sensitive biomarker assays.

Cardiomyocyte enriched miRs

MiR-1 and miR-133a represent the most abundantly expressed cardiac miRs and derive from two bicistronic miR clusters, miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1[25]. The latter cluster lies in intron 12 of mindbomb E3 ubiquitin protein ligase 1 (MIB1), a protein involved in apoptosis. MiR-1-1 and miR-1-2, as well as miR-133a-1 and miR-133a-2 are identical in humans[25,26]. MiR-133b, specifically expressed in skeletal muscle, differs from miR-133a in the 2 terminal nucleotides at the 3' end[25]. MiR-1 and miR-133 are essential for skeletal and cardiac muscle cell proliferation and differentiation[27]. In adult hearts, they exhibit an anti-hypertrophic effect[28].

Experimental MI in rodents[19,21,29,30] and in pigs[31] resulted in decreased expression of miR-1 and miR-133a in the infarcted area, and increased levels in whole blood or its components. Accordingly, human autopsy samples of infarcted tissue demonstrated downregulation of miR-1 and miR-133a/b[32]. Multiple reports[19-21,29-31,33-40] described significantly higher circulating levels of miR-1 and miR-133a in ACS compared to healthy controls, using either serum or plasma, either early or late (up to 24h) after MI. Plasma miR-1 and miR-133a/b levels paralleled those of conventional and high-sensitive Tn[30,35]. Areas under the curve (AUC) in receiver operating characteristics (ROC) curves ranged between 0.57 and 0.98 for miR-1[20,31,40] and 0.86 and 0.9468 for miR-133a[21,31,39]. Considerable overlap in miR-1 and miR-133a plasma levels in unstable angina (UA) and MI precluded their use for further subdivision of ACS[35]. Apart from serving as a biomarker for the diagnosis of ACS, circulating levels of miR-1 and miR-133a at 3 days (d) post-MI negatively correlated with renal function[31]. More importantly, their levels also associated with long-term major adverse cardiac events (MACE) and surrogate endpoints (infarct size, cardiac function and QRS duration) following MI. While miR-1 levels associated with QRS duration[20], infarct size[20,41] and negatively correlated with

systolic and diastolic cardiac function, especially in first MI[42], miR-133a predicted survival at 6 months post-MI[35], infarct size, reperfusion injury and decreased myocardial salvage following primary coronary intervention[43].

MiR-208a/b derive from the introns of the cardiac specific α- and β-myosin heavy chains (MYH6 and MYH7) respectively and are involved in stress-dependent cardiac growth and gene expression (fetal gene program)[44]. MiR-208 expression significantly increased in infarcted cardiac tissue compared to healthy tissue[32]. The suitability of miR-208a specifically as a circulating biomarker in CAD is controversial considering the contradictory information gathered: some considered miR-208a a reliable biomarker for stable CAD and ACS[19,34-36,45], whereas others failed to detect miR-208a in a considerable proportion of ACS patients studied and ultimately stopped verifying it as a biomarker[46,47]. Nonetheless, ROC curve analysis reportedly demonstrated AUC values of 0.965 for miR-208a in positive studies; for miR-208b, these values ranged between 0.82 and 1[31,39,40,48]. Importantly, when limiting the analysis to plasma samples obtained within 4 h after the onset of chest pain, miR-208a was more sensitive than conventional cardiac TnI to diagnose MI. MiR-208a's binding to cytosolic proteins readily released following cardiomyocyte damage might account for its earlier appearance in plasma compared to Tn. Comparable with miR-1 and miR-133a/b, miR-208b showed a large overlap between the different manifestations of ACS, hence making discrimination between UA and MI impossible with miR-208b[35,40]. In addition to providing diagnostic information, miR-208b also conveyed important prognostic information as it negatively correlated with ejection fraction (EF)[31,48], a composite of mortality or heart failure within 30 days of hospitalization[40] and all cause mortality at 6 months[35].

MiR-499 is located in an intron of β-myosin heavy chain 7b (MYH7b) and plays a role in myosin gene regulation. MiR-499 has its own promoter and alternative splicing can

uncouple miR-499 expression from MYH7b when coexpression is not required[49]. MiR-499's plasma levels consistently increased within 12-48 h after the onset of MI and its slower release kinetics caused its peak level to occur only 24h after coronary occlusion[30]. Reported AUC values ranged between 0.822 and 1[19,31,35-37,39,45,48,50]. Large overlap between its circulating levels in the different presentations of ACS precluded the use of miR-499 to pinpoint the exact ACS manifestation[35,40]. In geriatric patients, miR-499 allowed discrimination between non-ST elevation MI (NSTEMI) patients and acute HF patients[37]. Circulating levels of miR-499 also related to clinical outcomes and prognosis apart from delayed cardiomyocyte loss diagnosis as there was a weak negative correlation between miR-499 and EF in ST elevation MI (STEMI)[48]; moreover, miR-499 strongly associated with mortality or heart failure within 30 days of hospitalization[40].

Fibroblast enriched miRs

The genomic localization of miR-21 overlaps with the protein-coding gene transmembrane protein 49 (TMEM49 or vacuole membrane protein 1, VMP1), implicated in intracellular vacuole formation and cell death[51]. However, miR-21 has its own promoter, and its transcription occurs independently of TMEM49[52]. MiR-21 is well characterized as an important regulator of cardiac fibrosis[25,53]. In eldery NSTEMI patients (mean age > 80 years), miR-21 increased within 4-9h after the onset of symptoms[37], whereas in stable CAD[33] and type 2 diabetes mellitus (DM)[54], it decreased compared to healthy controls. However, in a large population study, miR-15, miR-29b, miR-126, miR-223 and miR-28-3p showed a stronger association with incident DM type 2 and miR-21 was abandoned to classify and predict DM[33,54].

Endothelial cell enriched miRs

MiR-126 is encoded by intron 7 of the EGF-like domain 7 gene, which acts as a chemoattractant and inhibitor of smooth muscle cell migration[55]. MiR-126 is specifically expressed in endothelial cells and is the most abundant endothelial miR[56]. It is selectively enriched in microvesicles released by apoptotic endothelial cells and upregulates endothelial CXCL12 and CXCR4 signaling in neighbouring cells, linking increased miR-126 expression to improved atherosclerotic plaque stability by enhanced progenitor cell influx, increased smooth muscle cell content and decreased macrophage content[24].

Impaired miR-126 packaging in microparticles and apoptotic bodies upon exposure to high glucose caused lower plasma miR-126 levels in type 2 DM compared to matched controls[54]. In line with this, patients with stable CAD had lower serum and plasma miR-126 compared to healthy controls[33]. However, two groups of patients diagnosed either with non-cardiac chest pain or stable CAD following coronary angiography had comparable circulating miR-126 levels; thus rendering its use as a biomarker for stable CAD controversial in a clinically more relevant situation[36,57]. In addition, higher aortic EDTA plasma levels and an increased transcoronary gradient of miR-126 in Tn positive acute coronary syndromes (ACS) suggested miR-126 consumption during transcoronary passage through the culprit vessel[36]. Intriguingly, one study measured decreased venous miR-126 plasma levels in MI patients compared to healthy controls up to one week following the event; corresponding AUC values to diagnose MI ranged between 0.83 and 0.88[38]. In the general population, plasma miR-126 positively associated with incident MI[58]. In ischemic HF, plasma miR-126 levels negatively correlated with New York Heart association (NYHA) score and brain natriuretic peptide (BNP) levels but increased upon clinical improvement[59]. However, the studied NYHA class III and IV patients had more diabetes and advanced

atherosclerosis and these confounding variables could also explain the observed downregulation of miR-126.

The miR-17-92 cluster (miR-17, 18a, 19a, 20a, 19b-1 and 92a-1) is located in an intergenic region and expression occurs from a single transcription unit forming a polycistron[25]. Plasma and serum miR-17 and miR-92a were significantly reduced in stable CAD compared to healthy controls[33], though analogous to miR-126, plasma levels were similar in patients with non-cardiac chest pain and stable CAD undergoing coronary angiography[36]. The latter study also demonstrated a significant increase in arterial plasma levels of miR-92a in Tn positive ACS patients compared to patients with stable CAD.

Inflammatory cell enriched miRs

MiR-155 maps within the B cell integration cluster (BIC) located on chromosome 21 and is to date one of the most extensively studied miRs with firmly established roles in neoplastic diseases, haematopoiesis, inflammation and both adaptive and innate immunity. Moreover, miR-155 regulates the renin-angiotensin system by its interaction with the 3' UTR of angiotensin 1 receptor (AT1R)[60,61]. Serum and EDTA-plasma levels of miR-155 were significantly lower in CAD patients compared to healthy volunteers[33]. However, miR-155 could not reliably differentiate between stable CAD and ACS in patients undergoing coronary angiography and its levels did not correlate with circulating hs Tn[36]. Interestingly, elevated serum miR-155 levels at the time of hospital discharge following MI predicted cardiac death within one year[62].

The miR-223 locus lies on the X chromosome and its transcription occurs independently of any known genes. It fine-tunes the generation and function of granulocytes[63]. The suitability of miR-223 as a robust biomarker seems dubious as one study showed a mild though significant decrease in its plasma levels in STEMI patients compared to patients with

atypical chest pain[45], whereas another trial discovered a trend towards an increase in its plasma levels in ACS compared to patients with stable CAD or no evidence of CAD[36]. In both studies, miR-223 did not correlate with circulating Tn levels.

Smooth muscle cell enriched miRs

MiR-143 and miR-145 are contained in a bicistronic cluster on the (non-coding) miR-143 host gene. They drive the differentiation from multipotent stem cells to smooth muscle cells and oscillations in their cellular concentrations alter the proliferative potential of vascular smooth muscle cells[64]. In acute STEMI, circulating miR-145 isolated from blood cells drawn 3.0 ± 2.3 h after the onset of symptoms, significantly increased and correlated with infarct size estimated by hs TnT release[65]. Patients with stable CAD had significantly reduced serum and EDTA plasma levels of miR-145[33].

Non-cardiac specific miRs

In addition to the aforementioned miRs examined in multiple trials, there are still others, typically non-cardiac or skeletal muscle specific, associated with CAD, ACS or post-MI prognosis validated in a few experiments at most. In general, three methodological approaches account for these observed differences. First, these peculiar miRs were specifically chosen. Second, rather than a priori selection of miRs (typically cardiac specific), few investigators started with a derivation cohort whose derived miRs they subsequently tested in a validation cohort. Third, whereas most research focused on cell-free miRs, only few studies used whole blood or peripheral blood mononuclear cells (PBMC) to detect miRs with biomarker potential.

Plasma miR-328, miR-30a and miR-195 significantly increased, while let-7b significantly decreased following MI compared to healthy subjects; furthermore, miR-328 peaked before

cardiac TnI[34,66]. Plasma miR-423-5p significantly increased in geriatric acute HF and NSTEMI patients[37]. Interestingly, elderly acute HF patients had the highest miR-423-5p levels, which corroborated an earlier report[67], yet contested by others[68,69]. In whole blood, miR-1291 and miR-663b showed the highest sensitivity and specificity for the discrimination of STEMI patients from controls (AUC 0.91 and 0.94 respectively). However, addition of other significantly dysregulated miRs created a 7 and 20 miR signature which predicted STEMI with even higher precision than any single miR (AUC 0.99) and outperformed hs TnT at early (< 4 h) time points[65,70]. Decreased plasma miR-150 levels at discharge predicted left ventricular (LV) remodeling following STEMI. Importantly, miR-150 outperformed N terminal-pro-brain natriuretic peptide (NT-pro-BNP) to predict LV remodeling (AUC 0.74 and 0.60)[71]. Serum miR-380* at discharge associated with cardiac death at 1 year following MI[62]. Whole blood miR-30c correlated with infarct size estimated by hs TnT[65].

In PBMC from CAD patients, the expression of miR-135a was 5-fold increased, while miR-147 was 4-fold decreased compared to healthy volunteers. Furthermore, miR-134, miR-198 and miR-370 differentiated UA from stable AP[72].

Treatment effects and influence of conventional cardiovascular risk factors

Few studies rigorously assessed the correlation between circulating miRs and conventional cardiovascular risk factors (age, gender, renal function, previous MI, hypertension and smoking status) in patients presenting with ACS[20,33,35,43,45]; furthermore, many restricted the correlation analysis to cardiomyocyte enriched miRs. Small sized studies typically ruled out any influence of the aforementioned clinical variables on the studied miR(s). These conclusions appeared valid for the study itself, however, the opposing conclusions from moderate sized studies seemed more reliably applicable to the general

population: significant univariate relations existed between miR-1 and renal function as well as smoking status; miR-133a correlated with age, previous MI and smoking; miR-499 associated with male gender. In multiple regression analyses, miR-1 independently associated with renal function, miR-133a with age and renal function, miR-208b with age and smoking and miR-499 with male gender[35]. Whereas total cholesterol, HDL and LDL moderately correlated with plasma and serum miR-155 in CAD, both age and renal function showed an inverse correlation. Furthermore, females CAD patients had significantly higher levels of circulating miR-155 compared to males.

Vasculoprotective therapies with statins, aspirin and inhibitors of the renin-angiotensin system (angiotensin converting enzyme inhibitors and angiotensin receptor blockers) significantly decreased plasma levels of endothelial cell (miR-126, miR-17-92 cluster), smooth muscle cell (miR-145) and inflammatory cell (miR-155) enriched miRs in a combined analysis of healthy volunteers and CAD patients. When restricting the analysis to CAD patients, only endothelial cell enriched miRs and statin therapy remained independently associated. Similarly, miR-133a decreased upon statin and aspirin treatment in a combined cohort of healthy volunteers and CAD patients, but the association disappeared when selectively analyzing CAD patients[33]. Highly controversial as other studies could only partially confirm these results[36,57], these findings nonetheless remain intriguing as they suggest that miRs could help in clinical practice to reliably gauge pleiotropic and protective effects of statins and other drugs routinely started following ACS and in CAD.

Whole blood gene expression profiling before and after a 10 weeks cardiac rehabilitation exercise program demonstrated increased circulating miR-92a and miR-92b levels and decreased levels of their targets involved in mitochondrial function and oxidative phosphorylation, indicative of a ‘healthier’ profile upon completion of the program[73].

Ready for prime time?

The assessment of the clinical potential of a novel cardiovascular biomarker is structured around three fundamental questions[74]. First, accurate measurements must be possible at a reasonable cost and with short turnaround times; second, the biomarker must add new and/or more accurate information; third, the biomarker must help the clinician in the management of patients.

Current quantification of miRs relies on polymerase chain reaction (PCR). PCR is readily available, easy to perform, sensitive and specific; however, results vary from day to day in contrast to ELISAs used for serum protein biomarkers, which are well established and have little day-to-day variation[75]. Furthermore, the complete process of miR isolation, reverse transcription and finally PCR itself takes several hours, thus new, preferably bedside tests need to be developed to make miRs more appealing for routine clinical use. In addition, the way of reporting circulating miR levels needs standardization. Nowadays, results are frequently reported as a fold change compared to an endogenous, supposedly unchanging miR, a spiked-in synthetic miR or the circulating level of the studied miR in a control group. However, analogous to other laboratory tests, reporting the absolute number of molecules per volume unit is feasible and more straightforward[29].

The experimental use of miRs as biomarkers in ACS coincided with the introduction of hs Tn assays in routine clinical practice, which allow definite MI diagnosis within three hours after presentation. In the small to medium-sized clinical studies performed to date, the diagnostic potential of selected cardiac enriched miRs using contemporary quantification tools approaches the sensitivity and specificity of hs Tn assays. However, these already executed trials used a plethora of biological materials (serum, plasma, whole blood, blood cells), and RNA isolation and quantitation tools. Large, multicenter, prospective trials with uniform sample handling and miR quantition are now mandatory in the first place to value the real

potential of miRs in comparison to contemporary Tn assays. Furthermore, aiming at substituting already clinically embedded hs Tn assays with miR based assays to diagnose ACS seems an unrealistic goal. Rather, developing miR based assays to make a diagnosis of ACS in selected clinical situations in which Tn based assays have a lower specificity seems a more reasonable goal, e.g. for the diagnosis of myocardial damage in patients with end stage renal disease, sepsis, myocarditis, or heart failure. Moreover, rather than focusing on one single or a set of miRs just to make a diagnosis of cardiomyocyte loss, profiling a set of miRs using current technologies should help to obtain additional invaluable information to tailor available treatments to each individual patient, as some miRs can inform about the duration of coronary occlusion, others give an indication of infarct size, still others about reperfusion injury and myocardial salvage. Apart from thus providing detailed information on the infarct burden itself, miRs might also help to identify those patients at higher risk for complications of ACS such as renal impairment, arrhythmogenesis and heart failure, justifying intensified in hospital monitoring and/or post-discharge follow-up. In the case of heart failure, distinct miR profiles might provide enough diagnostic information to diagnose its etiology, avoiding invasive procedures with their inherent risks. Thus, rather than aiming to replace the more familiar Tn based assays, we should embrace miRs and their potential to provide complementary information on infarct and reperfusion characteristics, as well as short term and long term repercussions on distant organs and the whole organism.

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In conclusion, miRs are promising biomarkers for symptomatic and asymptomatic CAD providing direct understanding of detrimental pathophysiological processes in which they are themselves crucially involved. As such, they provide valuable diagnostic and prognostic information about CAD and ACS. Importantly, rather than aiming at substituting available

biomarkers for ACS (Tn) and HF (NT-pro-BNP), the community should focus on supplementing these with miR profiles to obtain complementary information.

However, several challenges still lie ahead before their routine clinical application. First, essential knowledge on their biochemical composition in blood is still lacking. In addition, some technical issues need to be resolved to make miRs more attractive for routine clinical use of which shorter turnaround times, increased sensitivity of assays and adequate standardization of extraction and quantitation are the most urgent ones. Most importantly, large, multicenter and prospective trials need to be initiated to assess the real diagnostic value of miRs.

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Disclosures

None.

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Figure Legends

Figure 1. Circulating miRs as biomarkers in stable CAD, DM type 2 (top) and ACS (bottom). Overview of miRs whose circulating levels were repeatedly shown to be altered in the indicated clinical conditions.

Figure Legends

Figure 1. Circulating miRs as biomarkers in stable CAD, DM type 2 (top) and ACS (bottom). Overview of miRs whose circulating levels were repeatedly shown to be altered in the indicated clinical conditions.

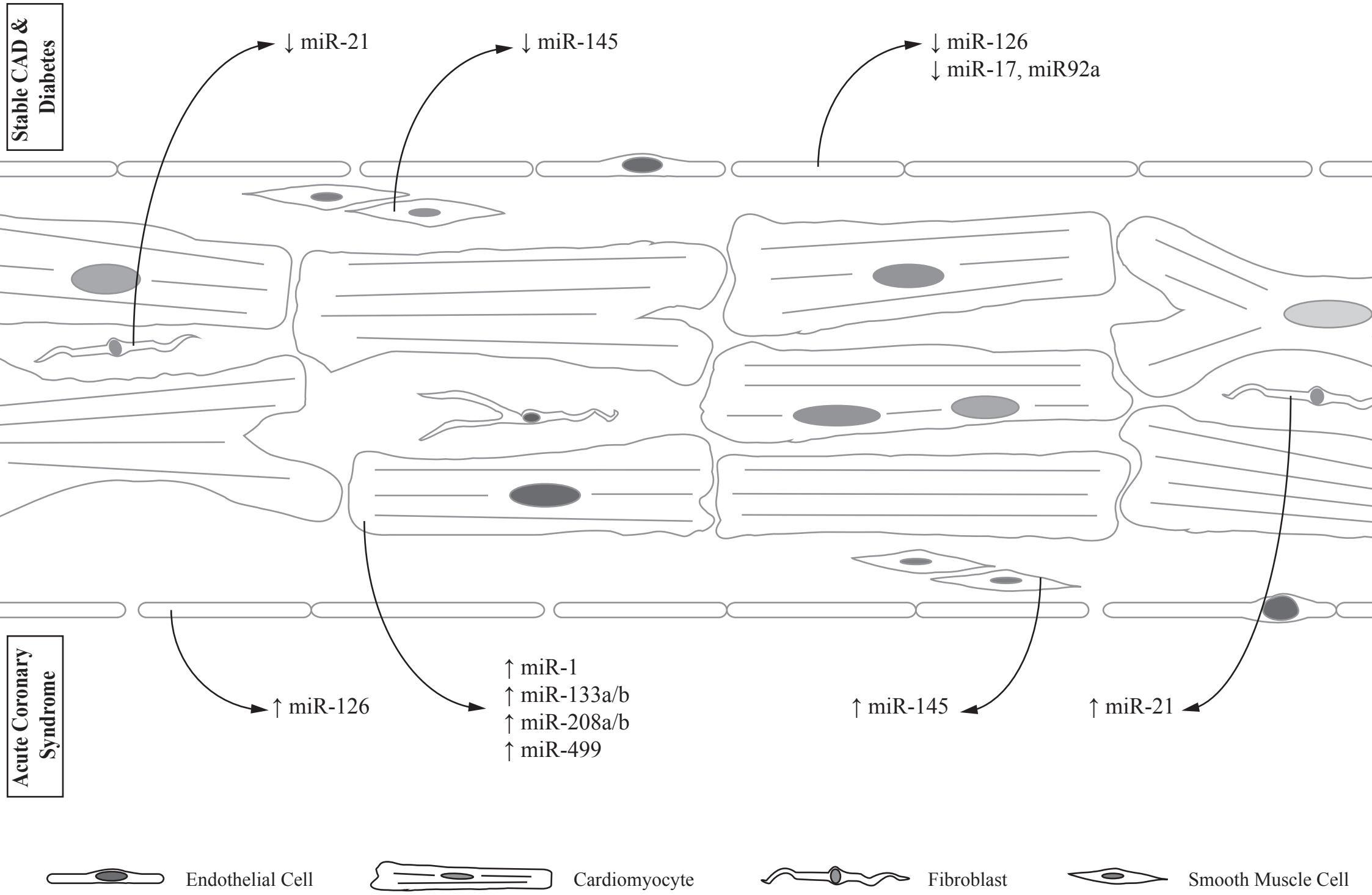
Stable CAD & Diabetes
↓ miR-21
↓ miR-145
↓ miR-126
↓ miR-17, miR92a
Acute Coronary Syndrome
↑ miR-126
↑ miR-1
↑ miR-133a/b
↑ miR-208a/b
↑ miR-499
↑ miR-145
↑ miR-21
Endothelial Cell
Cardiomyocyte
Fibroblast
Smooth Muscle Cell

Table 1: Studies with miRs as biomarkers for asymptomatic CAD and DM type 2.

Disease	Specimen	Sample size	Main findings	Ref.
Stable CAD	EDTA-Plasma (derivation cohort); serum (validation cohort)	Derivation cohort: n(CAD) = 36 ; n(Ctrl) = 17. Validation cohort: n(CAD) = 31 ; n(Ctrl) = 14.	Derivation cohort: ↓ miR-126, miR-17, miR-92a, miR-199a, miR-155, miR-145. ↑ miR-133a. Validation cohort: ↓ miR-126, miR-17, miR-92a, miR-155, miR-145.	[33]
Type 2 DM	Plasma	n(DM) = 80 ; n(Ctrl) = 80. Bruneck cohort, n = 822	↓ miR-24, miR-21, miR-20b, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, miR-486 ↓ miR-126 in DM	[54]
Stable CAD	Plasma	n(CAD) = 31 ; n(Ctrl) = 36	Failure to repeat observation of dysregulated miR-126 in CAD. MiR-126 inversely associated with LDL cholesterol in CAD patients. MiR-126 positively associated with LDL cholesterol in Ctrl patients.	[57]
Type 2 DM	Plasma	Bruneck cohort, n = 820	MiR-126 associated with incident MI. MiR-223 and miR-197 inversely associated with future MI.	[58]
(Un)stable CAD	Whole blood	n(SAP) = 25 [stable CAD] ; n(UAP) = 25 [unstable CAD] ; n(Ctrl) = 20	↑ miR-135a, ↓ miR-147 in CAD vs Ctrl. MiR-142-3p, miR-223, miR-150, miR-21, miR-16 and let-7a not significantly different between CAD and Ctrl.	[72]

			↑ miR-134, miR-370, ↓ miR-198 in UAP vs SAP	
Stable CAD	Whole blood	n(CAD) = 12 ; n(Ctrl) = 12	↑ miR-140-3p, miR-182	[73]
			365 differentially expressed genes: 175 genes upregulated; 190 genes down-regulated.	
		10 CABG patients before and after cardiac rehabilitation	↑ miR-92a, miR-92b	
			645 differentially expressed genes: 196 genes upregulated; 449 genes downregulated.	

Abbreviations: CABG: coronary artery bypass grafting; CAD: coronary artery disease; Ctrl: Controls; DM: diabetes mellitus; MI: myocardial infarction; miR: microRNA; Ref.: Reference; SAP: stable angina pectoris; UAP: unstable angina pectoris.

Table 2: Studies with miRs as biomarkers for ACS.

Disease	Specimen	Sample size	Main findings	Secondary findings	Ref.
MI (< 12h)	Plasma	n(MI) = 33 ; n(non-MI chest pain) = 33 ; n(Ctrl) = 30	↑ miR-1, miR-133a, miR-499, miR-208a	AUC(miR-208a) = 0.965 ; AUC(miR-499) = 0.822 ; AUC(miR-1) = 0.847 ; AUC(miR-133a) = 0.867 AUC(TnT) = 0.987	[19]
MI	Citrate-Plasma	n(AMI) = 93 ; n(Ctrl) = 66.	↑ miR-1	AUC(miR-1): 0.7740 MiR-1 levels correlated with QRS duration.	[20]
ACS (< 24h; 7/29 undetermined)	Serum	n(ACS) = 29 ; n(non-ACS) = 42	↑ miR-1 , miR-133a	MiR-208a detectable in a few patients only. ↑ MiR-1 and miR-133a in UA and apical ballooning. AUC (miR-1) = 0.777 ; AUC(miR-133a) = 0.932	[21]
MI (< 24h)	Serum	n(MI) = 31 ; n(Ctrl) = 20.	↑ miR-1	Positive correlation with CK-MB levels (r=0.68).	[29]
STEMI (< 14h)	EDTA-Plasma	n(STEMI) = 33 (25 + 8) ; n(Ctrl) = 17.	↑ miR-1, miR-133a, miR-133b, miR-499-5p. ↓ miR-122, miR-375.	↑ miR-208a could not be reproduced. MiR-1, miR-133a/b peak occurred before TnI. Slower kinetics of miR-499: peak after 24h.	[30]
STEMI (< 24h)	Plasma	n(STEMI) = 25 ; n(Ctrl) = 11	↑ miR-1, miR-133a, miR-208b, miR-499-5p	miR-1 and miR-133 detectable in urine. AUC in STEMI < 12 h; AUC(miR-208b) = 1 ; AUC(miR-1) = 0.98 ; AUC(miR-133a) = 0.86 ; AUC(miR-499-5p) = 0.99	[31]

				MiR-1 (r^2 = 0.27) and miR-133a (r^2 = 0.17) levels at day 3 negatively correlated with GFR. Peak values of miR-208b negatively correlated with EF (r^2 = 0.32) and positively correlated with peak levels of TnT (r^2 = 0.25) at day 1.	
MI (< 24h)	EDTA-Plasma and whole blood	n (AMI) = 51 ; n(Ctrl) = 28	↑ miR-133 and miR-328 in plasma and whole blood.	Plasma: AUC(miR-133) = 0.890 ; AUC(miR-328) = 0.810 Whole blood: AUC(miR-133) = 0.702 ; AUC(miR-328) = 0.872	[34]
ACS	Plasma	n(UA) = 117 ; n(NSTEMI) = 131 ; n(STEMI) = 196.	↑ miR-1, miR-133a, miR-208b in (N)STEMI compared with UA.	No association with arrhythmias detected. miR-133b, miR-208a, miR-499 not elevated in MI compared to UA. Considerable overlap between the three diagnostic groups. miR-133a and miR-208b associated with risk of death.	[35]
ACS and CAD	EDTA-Plasma	n(ACS) = 19 ; n(CAD) = 31 ; n(no CAD) = 7	↑ miR-499, miR-133a, miR-208a in ACS vs. CAD.	Transcoronary decrease of miR-126 and miR-92a suggestive of consumption by the culprit artery.	[36]

			↑ in miR-92a, miR-126.	Transcoronary increase of miR-133a and miR-499.	
NSTEMI in the eldery (< 9h)	EDTA-Plasma	n(NSTEMI) = 92 ; n(acute CHF) = 81 ; n(Ctrl) = 99	↑ miR-133a, miR-423-5p, miR-21, miR-499-5p in NSTEMI and acute CHF vs Ctrl. ↑ miR-21 and miR-499-5p in NSTEMI vs acute CHF. ↑ miR-423-5p in acute CHF vs NSTEMI.	AUC(miR-499-5p) = 1 in NSTEMI AUC(cTnT) = 1 in NSTEMI AUC(miR-499-5p) = 0.88 in acute CHF AUC(cTnT) = 0.78 in acute CHF AUC(miR-499-5p) = 0.88 in NSTEMI vs acute CHF AUC(cTnT) = 0.93 in NSTEMI vs CHF AUC(miR-499-5p) = 0.86 ; AUC(cTnT) = 0.68 ; AUC(hs-TnT) = 0.70 to distinguish NSTEMI from acute CHF with mild elevation of cTnT (< 0.10 ng/mL).	[37]
MI (+/- 4h + time series)	Plasma	n(MI) = 17 ; n(Ctrl) = 25.	↑ miR-1 up to one week after MI. ↓ miR-126 up to one week after MI.	AUC(miR-1) = 0.90 – 0.96 AUC(miR-126) = 0.83 – 0.88	[38]
MI (< 12h)	EDTA-Plasma	n(MI) = 67 ; n(Ctrl) = 32.	↑ miR-1, miR-133a, miR-208b, miR-499	No significant differences in the levels of these four miRs between STEMI and NSTEMI. AUC(miR-1) = 0.8265 ; AUC(miR-133a) = 0.9468 ; AUC(miR-208b) = 0.8899 ; AUC(miR-499) = 0.8841 ;	[39]

				AUC(cTnT) = 0.9820. None of four miRs tested superior to cTnT for MI diagnosis.	
MI (< 1 week)	EDTA-Plasma	n(STEMI) = 173 ; n(NSTEMI) = 146 ; n(Ctrl) = 88.	↑ miR-208b, miR-499-5p in (N)STEMI. ↑ miR-1 in STEMI	Diagnostic accuracy for MI: AUC(miR-1) = 0.57 , AUC(miR-208b) = 0.82 , AUC(miR-499-5p) = 0.79 ; AUC(hs TnT) = 0.95. MiR-208b (OR: 1.79) and miR-499-5p (OR: 1.70) associated with compound of death < 30 days, heart failure, EF < 40% or cardiogenic shock. Prognostic accuracy: AUC(miR-208b) = 0.64 , AUC(miR-499-5p) = 0.64 ; AUC(hs TnT) = 0.66. Strong correlation with hs TnT of miR-208b (r = 0.65) and miR-499-5p (r = 0.62). Weak negative correlation with LVEF of miR-1 (r = -0.11), miR-208b (r = -0.16) and miR-499-5p (r = -0.16).	[40]
MI	Plasma	n(MI) = 49	miR-1 negatively correlates with systolic and diastolic function	MiR-1 negatively correlates with EF (r = -0.7605), FS (r = -0.5120) and E/A (r = -0.6862). Result driven by first MI subgroup.	[42]

STEMI (<12h)	Serum	n(STEMI) = 216	↑ miR-133a associated with decreased myocardial salvage, larger infarcts, more pronounced reperfusion injury.	If miR-133a ≥ median more likely to have anterior infarction, diabetes, higher Killip class, longer time from symptom onset to reperfusion, higher peak CK levels, higher frequency of incomplete ST-segment resolution < 70% after reperfusion, more reduced EF. Death, reinfarction, new CHF within 6 months after infarction higher in miR-133a ≥ group.	[43]
MI (< 12h)	Citrate-Plasma	n(AMI) = 32 ; n(Ctrl) = 36.	↑ miR-133a, miR-208b, miR-499 ↓ miR-223.	AUC(miR-208b): 0.944 AUC(miR-499): 0.918 Correlation with TnT and CK levels of miR-499 (r = 0.69 resp. r = 0.41) and miR-208b.	[45]
MI (< 12h)	Citrate-Plasma	n(STEMI) = 397 ; n(NSTEMI) = 113 ; n(Ctrl) = 87	↑ miR-208b, miR-499 in both STEMI and NSTEMI vs. Control.	Higher miR-208b and miR-499 levels in STEMI vs NSTEMI patients.. AUC(miR-499) = 0.97 ; AUC(miR-208b) = 0.90 ; AUC(hs-cTnT) = 0.97	[48]
ACS (< 48h)	EDTA-Plasma	n(AMI) = 9 ; n(UA) = 5 ; n(CHF) = 15 ; n(Ctrl) = 10.	↑ miR-499	MiR-499 correlated with CK-MB.	[50]
Ischemic heart disease , CHF	EDTA-Plasma	n(ischemia) = 33 ; n(Ctrl) = 17.	↓ miR-126 in CHF	Failure to repeat observation of increased miR-423-5p in CHF[67].	[59]

MI	Serum	Derivation cohort: n(cardiac death | MI) = 7 ; n(event free | MI) = 7. Validation cohort: n(cardiac death | MI) = 19 ; n(event free | MI) = 21.	11 miRs differently expressed. 2 miRs differently expressed.	↑ miR-134, miR-155, miR-18a, miR-192, miR-380* ↓ miR-125-5p, miR-212, miR-331-3p, miR-223*, miR-190b, miR-93*. ↑ miR-308*, miR-155 in cardiac death group.	[62]
MI	Whole blood	n(MI) = 20 ; n(Ctrl) = 20.	↑ miR-663b, miR-1291 and 119 other miRs. Creation of miR signature to predict MI.	Creation of miR signature consisting of 20 miRs to predict MI (specificity 96%, sensitivity 90%, and accuracy 93%). AUC(miR-663b) = 0.94 ; AUC(miR-1291) = 0.91. MiR-30c and miR-145 correlate with infarct sizesestimated by TnT release.	[65]
MI (+/- 4h + time series)	Plasma	n((MI) = 18 ; n(Ctrl) = 30.	↑ miR-30a 4-12 h after MI; ↑ miR-195 8-12h after MI. ↓ let-7b up to one week after MI.	AUC(miR-30a) = 0.87 – 0.89 AUC(miR-195) = 0.88 – 0.89 AUC(let-7b) = 0.85 – 0.89 AUC(miR-30a + miR-195 + let-7b) = 0.92 – 0.93	[66]
MI	Whole blood	n(MI) = 18 ; n(Ctrl) = 21.	↑ miR-181c* ↓ miR-1915, miR-339-3p Creation of miR signature to	Creation of miR signature consisting of 7 miRs to predict MI; highest discriminative power at presentation (0h) and 2h after presentation, with	[70]

			predict MI.	AUC(0h) = 0.89 ; AUC(2h) = 0.92.	
MI	Plasma	n(STEMI) = 60	Identification of 10 miRs predicted to regulate LV remodeling.	miR-27a, miR-133a, miR-625, miR-296-3p, miR-31, miR-23a, miR-204, miR-19a, miR-101, miR-150.	[71]
		n(STEMI) = 30	↓ miR-150 predicts LV remodeling	AUC(miR-150) = 0.74 AUC(NT-pro-BNP) = 0.60 AUC(miR-150 + NT-pro-BNP) = 0.77 MiR-150 reclassified 54% of patients misclassified by NT-pro-BNP and 59% of patients misclassified by multi-parameter clinical model.	

Abbreviations: ACS: acute coronary syndrome; AUC: area under the curve; CAD: coronary artery disease; CHF: congestive heart failure; CK: creatine kinase; Ctrl: Controls; DM: diabetes mellitus; EF: ejection fraction; FS: fractional shortening ; GFR: glomerular filtration rate; h: hours ; hs: high sensitive ; MI: myocardial infarction; miR: microRNA; NSTEMI: non-ST elevation myocardial infarction; NT-pro-BNP: N-terminal fragment of pro-brain natriuretic peptide; OR: Odds Ratio ; Ref.: Reference; STEMI: ST elevation myocardial infarction; Tn: Troponin; UA: unstable angina.